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Tubules are the major site of M-CSF production in experimental kidney disease: Correlation with local macrophage proliferation[1]

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Tubules are the major site of M-CSF production in experimental kidney disease: Correlation with local macrophage proliferation.

Background. Local proliferation of macrophages occurs within both the glomerulus and the interstitium in severe forms of human and experimental glomerulonephritis and plays an important role in amplifying renal injury. Macrophage colony-stimulating factor (M-CSF) is thought to be the growth factor driving this local macrophage proliferation. Previous studies have found that glomeruli are the predominant source of M-CSF production. However, this is difficult to reconcile with the prominent macrophage accumulation and proliferation seen in the interstitial compartment in glomerulonephritis. To address this issue, we localized M-CSF expression in rat models of glomerular versus tubulointerstitial injury and examined its relationship to local macrophage proliferation.

Methods. M-CSF expression (Northern blotting, in situ hybridization, immunostaining, Western blotting) and local macrophage proliferation (double immunostaining) was examined in normal rat kidney on days 1 and 14 of rat anti-glomerular basement membrane (anti-GBM) glomerulonephritis and on day 5 following unilateral ureteric obstruction.

Results. M-CSF mRNA and protein expression were identified in small numbers of glomerular podocytes, approximately 25% of cortical tubules, and most medullary tubules in normal rat kidney. Northern blotting showed a significant increase in whole kidney M-CSF mRNA in rat anti-GBM glomerulonephritis. Up-regulation of glomerular and, most prominently, tubular M-CSF production was confirmed by three independent methods: in situ hybridization, immunostaining, and Western blotting. The increase in M-CSF expression colocalized with local macrophage proliferation (ED1+PCNA+ cells) in both the glomerulus and tubulointerstitium. On day 5 after ureter ligation, there was a significant increase in tubular M-CSF mRNA and protein expression in the obstructed kidney, with no change in glomerular M-CSF. In parallel with M-CSF expression, macrophage accumulation and proliferation was prominent in the interstitium, but was absent from glomeruli.

Conclusions. The tubular epithelial cell is the major site of M-CSF production within the injured kidney. Indeed, substantial macrophage accumulation and local proliferation can occur in the tubulointerstitium in the absence of glomerular inflammation. These results suggest that M-CSF production within the kidney, particularly by tubular epithelial cells, plays an important role in regulating local macrophage proliferation in experimental kidney disease.

The accumulation of macrophages in glomeruli and the interstitium is a common feature in most types of glomerulonephritis [1]. There is a highly significant correlation between the intensity of macrophage accumulation and the degree of renal dysfunction and histologic damage [1–3]. Indeed, interstitial macrophage accumulation is predictive of disease progression in lupus nephritis and IgA nephropathy [4, 5].

Recent studies have shown that local proliferation is an important mechanism of macrophage accumulation in severe forms of human and experimental glomerulonephritis [2, 6–10]. This has been demonstrated by the expression of Ki-67 or proliferating cell nuclear antigen (PCNA), incorporation of bromodeoxyuridine (BrdU), and the presence of mitotic figures in combination with immunohistochemistry staining of macrophages [2, 6–10]. Local macrophage proliferation, most notably in the interstitium, correlates with the degree of renal dysfunction and histologic damage in human and experimental glomerulonephritis, indicating that this is an important mechanism for amplifying macrophage-mediated renal injury [2, 7, 8].

A number of studies suggest that macrophage colony-stimulating factor (M-CSF) is the main growth factor driv-

[1] **See Editorial by Rovin, p. 797**

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ing local macrophage proliferation in the kidney. M-CSF is best known as the pivotal growth factor regulating monocyte/macrophage proliferation and survival, although roles for M-CSF in osteoclast formation and implantation of the blastocyst into the endometrium and thrombocytopenia have also been described [11]. A lack of M-CSF production in the *op/op* mouse leads to a profound monocytopenia and substantially reduced tissue macrophage populations, which is a situation that can be reversed by the administration of M-CSF [11, 12]. M-CSF is also chemotactic for blood monocytes, and M-CSF has been shown to induce proliferation of a subset of human blood monocytes in vitro [13, 14]. Systemic administration of M-CSF enhanced glomerular macrophage accumulation and proteinuria in endotoxin-induced renal injury in mice [15]. Local administration of M-CSF via implantation of transfected cells under the kidney capsule induced tubulointerstitial macrophage infiltration and histologic damage in MRL-*lpr* lupus-prone mice [16]. In addition, M-CSF as an adjunct to chemotherapy in a patient with acute myeloblastic leukemia caused nephrotic syndrome with marked glomerular macrophage accumulation [17]. Thus, M-CSF may promote both the recruitment and local proliferation of blood monocytes within the injured kidney.

Macrophage colony-stimulating factor is predominantly expressed in the glomerulus in MRL-*lpr* lupus-prone mice and in human IgA nephropathy, with little production seen in the tubulointerstitium [18, 19]. The relative lack of M-CSF production in the tubulointerstitium is difficult to reconcile with the fact that the majority of macrophage infiltration and local proliferation occurs in the interstitium. There are three possible explanations for this apparent contradiction. First, M-CSF production within the injured glomerulus may diffuse down the mesangial stalk into the interstitium and promote the recruitment and local proliferation of monocytes from peritubular capillaries. Such a mechanism has been postulated to account for the induction of hilar and periglomerular macrophage infiltration in the very early stages of rat anti-glomerular basement membrane (anti-GBM) glomerulonephritis [20]. Second, M-CSF production in the tubulointerstitium may have been underestimated in the small number of studies that have addressed this issue. Third, interstitial macrophage accumulation may be independent of M-CSF.

This study examined M-CSF expression in two rat models of kidney disease: anti-GBM glomerulonephritis and unilateral ureteric obstruction. The aims of the study were to (*1*) localize M-CSF expression in models of glomerular versus tubulointerstitial injury and (*2*) examine the relationship between M-CSF expression and local macrophage proliferation in both the glomerulus and tubulointerstitium.

METHODS

Animals and reagents

Inbred male Sprague-Dawley (SD) rats (150 to 200 g) and inbred Wistar rats (150 to 200 g) were obtained from Monash Animal Services (Melbourne, Australia). Cytokines used were recombinant human and mouse M-CSF (R&D Systems, Inc., Minneapolis, MN, USA) and recombinant mouse interleukin-1α (IL-1α; Promega, Madison, WI, USA). Monoclonal antibodies (MoAbs) used were ED1, anti-rat CD68 (macrophage-specific antigen) [21, 22], and PC-10, anti-PCNA, which labels cells in the G_1, S, and G_2 phases of the cell cycle [23, 24]. Peroxidase-conjugated goat anti-mouse IgG, peroxidase-conjugated rabbit anti-guinea pig IgG, mouse peroxidase anti-peroxidase complexes (PAP), alkaline phosphatase-conjugated goat anti-mouse IgG, and alkaline phosphatase-conjugated mouse anti-alkaline phosphatase complexes (APAAP) were purchased from Dako Australia Pty. Ltd. (Botany, NSW, Australia).

Experimental anti-GBM glomerulonephritis

Accelerated anti-GBM glomerulonephritis was induced in SD rats by immunization with 5 mg normal sheep IgG in Freund's complete adjuvant followed by an intravenous injection of sheep anti-rat GBM serum (10 mL/kg body wt) five days later (termed day 0). Groups of five animals were killed on days 1 and 14. An additional group of five animals was killed on day 14 for preparation of tubular lysates. A group of five normal SD rats was also examined. Protein excretion in 24-hour urine collections on days 1, 7, and 14 was determined using the benzethonium chloride method. Concentrations of plasma and urine creatinine were measured using the standard Jaffe rate reaction (alkaline picrate method).

Tissues for histology were fixed in 10% formalin, and 4 μm paraffin sections were stained with the periodic acid-Schiff reagent or hematoxylin and eosin. The percentage of glomeruli exhibiting glomerular crescent formation was assessed by examination of at least 100 glomerular cross-sections per animal. The whole cortical tubulointerstitium was assessed for damage (tubular atrophy, leukocytic infiltration, and fibrosis) and scored as follows: 0 = no damage; 1 = damage of up to 25% of the cortex; 2 = damage of 25 to 50%; and 3 = damage of more than 50% of the cortex.

Unilateral ureter obstruction

A midline incision was made in anesthetized Wistar rats, and the left ureter was located. Two ties were made using 4.0 silk, and then the ureter was cut between the ties to avoid retrograde urinary tract infection. A group of five rats was killed five days after surgery. The right kidney was used as the nonobstructed control. A group of normal Wistar rats was also examined.

Preparation of anti–M-CSF antibody

A 720 bp cDNA fragment encoding the extracellular N-terminal region (nucleotides 244–923) of the mature rat M-CSF protein [25] was amplified by reverse transcription polymerase chain reaction (PCR) using the primers 5′-ACTAGTGGATCCAAGGAGGTGTCAG AACACTGTAGCCA-3′ containing a *Bam HI* restriction site and 5′-CATGTCGAATTCTCATGACTCGA GGGTCTGGCAGGTACTCC-3′ containing an *Eco RI* restriction site and a stop codon. The PCR product was cloned using the pMOSBlue T-vector kit (Amersham International, Buckinghamshire, UK) and was verified by DNA sequencing. Rat M-CSF cDNA was subcloned into the pRSET A vector (Invitrogen, San Diego, CA, USA) using *Eco RI* and *Bam HI* and expressed as a His-tag fusion protein in *E. coli* strain BL21(DE3)pLysS using the Xpress system (Invitrogen). Recombinant rat M-CSF was insoluble and purification required solubilization in 0.1% sodium dodecyl sulfate (SDS) followed by Ni-column affinity chromatography. A polyclonal antibody was raised by repeated immunization of outbred English Shorthaired guinea pigs with recombinant rat M-CSF in Freund's adjuvant. Affinity chromatography (Avid-AL gel; Haem, Melbourne, Australia) was used to purify the IgG fraction of guinea pig anti–M-CSF antiserum. The IgG fraction of normal guinea pig serum was purified as an isotype control.

Probes

A 358 bp cDNA fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was prepared by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into the pMOSBlue vector (Amersham International). Sense and antisense riboprobes for rat M-CSF and GAPDH were labeled with digoxigenin (DIG)-uridine triphosphate (UTP) using a T7 RNA polymerase kit (Roche Biochemicals, Mannheim, Germany). Incorporation of the DIG label was determined by dot blotting.

Northern blotting

A half kidney was dissected from normal and diseased rats, roughly diced, and then snap-frozen in liquid nitrogen. Tissues were ground into a powder using a metal mortar and pestle on dry ice. Total cellular RNA was extracted from the frozen tissue powder using the TRIzol reagent (GIBCO BRL, Gaithersburg, MD, USA). Alternatively, cultured cells were lysed directly in TRIzol and RNA extracted.

Northern blotting was performed as previously described [26]. RNA samples (15 μg) were denatured with glyoxal and dimethylsulphoxide, size fractionated on 1.2% agarose gels, and capillary blotted onto positively charged nylon membranes (Roche Biochemicals). Membranes were hybridized overnight with DIG-labeled cRNA probes at 68°C in DIG Easy Hyb solution (Roche Biochemicals). Following hybridization, membranes were washed finally in 0.1 × standard saline citrate (SSC)/0.1% SDS at 68°C. Bound probes were detected using sheep anti-DIG antibody (Fab) conjugated with alkaline phosphatase and development with CPD-star enhanced chemiluminescence, which was captured on Kodak XAR film. Densitometric analysis used the Gel-Pro Analyzer program (Media Cybernetics, Silver Spring, MD, USA).

In situ hybridization

In situ hybridization was performed as previously described, with some modification [27]. Formalin-fixed paraffin sections (4 μm) were heated in a microwave oven for 10 minutes as outlined previously in this article, incubated with 0.2 mol/L HCl for 15 minutes, followed by 1% Triton X-100 for 15 minutes, and then digested for 20 minutes with 10 μg/mL proteinase-K at 37°C (Roche Biochemicals). Sections then were washed in 2 × SSC, prehybridized, and then hybridized with 0.3 ng/μL DIG-labeled sense or antisense M-CSF cRNA probe overnight at 42°C in a hybridization buffer containing 50% deionized formamide, 4 × SSC, 2 × Denhardt's solution, 1 mg/mL salmon sperm DNA, and 1 mg/mL yeast tRNA. Sections were washed finally in 0.1 × SSC at 42°C, and the hybridized probe was detected using alkaline phosphatase-conjugated sheep anti-digoxigenin IgG and color development with NBT/BCIP (Roche Biochemicals).

Immunohistochemistry staining

Sections (4 μm) of formalin-fixed, paraffin-embedded tissues were dewaxed, rehydrated, and incubated for 20 minutes in 10% fetal calf serum (FCS) and 10% normal rabbit serum, followed by incubation with 10% bovine serum albumin (BSA) for 10 minutes. Sections were incubated overnight with guinea pig anti–M-CSF antibody at 4°C. Sections then were washed three times with phosphate-buffered saline (PBS)/0.05% Tween 20 and endogenous peroxidase blocked by incubation in methanol containing 0.3% H_2O_2 for 20 minutes. Sections were then incubated with peroxidase-conjugated rabbit anti-guinea pig IgG, washed three times in PBS/0.05%Tween 20, and then the signal was amplified using the Biotinyl Tyramide kit using streptavidin-conjugated peroxidase (TSA-Indirect kit; NEN Life Science Products, Boston, MA, USA). The bound peroxidase was developed with diaminobenzidine to produce a brown color. Negative controls included the use of normal guinea pig IgG in place of the anti–M-CSF antibody, omission of the primary or secondary antibody, and blocking the staining reaction by preincubation of the anti–M-CSF antibody with a 20-fold excess of recombinant human M-CSF.

Macrophage proliferation was detected by two-color immunohistochemistry staining using a microwave-based technique as previously described [28]. Formalin-fixed

(4 μm) paraffin sections were dewaxed, rehydrated through graded alcohols, and treated with microwave oven heating for 10 minutes in 0.01 mol/L sodium citrate, pH 6.0, at 2450 MHz and 800 W. Next, sections were preincubated with 10% FCS and 10% normal goat serum in PBS for 20 minutes, drained, and labeled with the ED1 MoAb for 60 minutes. After washing in PBS, endogenous peroxidase was inactivated by incubation in 0.3% H_2O_2 in methanol, and then sections were labeled with peroxidase-conjugated goat anti-mouse IgG followed by mouse PAP and developed with diaminobenzidine to produce a brown color. Sections were microwave heated a second time to denature bound immunoglobulin molecules, which prevented antibody cross-reaction [28]. This was followed by a preincubation step as described previously and then incubation overnight with the PC-10 MoAb. After washing in PBS, sections were labeled sequentially with alkaline phosphatase-conjugated goat anti-mouse IgG and mouse APAAP and were developed with Fast Blue BB Base (Sigma Chemical Co., St. Louis, MO, USA). As a negative control, an isotype-matched irrelevant MoAb (73.5; mouse anti-human CD45R) was used in place of one or both primary antibodies.

Quantitation of immunohistochemistry

Glomerular M-CSF staining was assessed in 50 glomerular cross-sections (gcs) per animal under a high-power (×400) in a semiquantitative fashion as follows: 0 = no labeling; 1 = 1 to 15 cells positive; 2 = 15 to 30 cells positive; and 3 = >30 cells positive. Tubular M-CSF staining was scored in 20 consecutive medium power (×200) fields by means of a 0.02 mm^2 graticule fitted in the eyepiece of the microscope. Fields progressed from the outer to inner cortex and back to the outer cortex, avoiding only large vessels and glomeruli, allowing assessment of at least 500 tubular cross-sections per animal. A positive tubular cross-section was defined as having two or more stained cells.

The number of ED1+ macrophages and ED1+PCNA+ proliferating macrophages was assessed on double-stained tissue sections under high power (×400). Only ED1+ macrophages in which the nucleus was evident were counted and assessed for the presence of nuclear PCNA staining. Note that this method of counting underestimates the total number of macrophages. The number of glomerular ED1+ and ED1+PCNA+ cells were counted in 50 gcs per animal. The number of interstitial ED1+ and ED1+PCNA+ cells were counted in 100 consecutive high-power (×400) cortical fields as described previously in this article, and expressed as cells per mm^2 with no correction made for the tubular area. All scoring was performed on coded slides.

Tubular lysates

Animals with anti-GBM disease were killed on day 14, and the kidneys were removed. The tissue was finely minced, and differential sieving was performed to remove glomeruli. The tubular fragments were then lysed in 1 mL of RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and a proteinase inhibitor cocktail (P2714; Sigma-Aldrich Pty., Sydney, NSW, Australia). The protein concentration was determined by the Coommassie Protein Assay Reagent (Pierce, Rockford, IL, USA). Samples were aliquoted and stored at −80°C.

Cell lysates

Cell lines used were the 1097 rat mesangial cell line isolated from SD rats [29] and the NRK52E normal rat tubular epithelial cell line (American Type Culture Collection, Manassas, VA, USA). One thousand ninety-seven cells were cultured in RPMI 1640 medium (GIBCO) with 10% heat-inactivated FCS, 10 mmol/L HEPES buffer, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37°C. NRK52E cells were cultured in the same medium supplemented with 1% nonessential amino acids. Cells were grown in 175 cm^2 flasks until confluent, washed (×3) with PBS, and then solubilized in lysis buffer [25 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), and a proteinase inhibitor cocktail] for 30 minutes on ice. Samples were centrifuged at 14,000 × *g* for five minutes to pellet cell debris and were aliquoted and stored at −80°C.

Western blotting

Samples were mixed with an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for five minutes, and electrophoresed on a 10% SDS polyacrylamide gel. Proteins were transferred onto Hybond-ECL nitrocellulose membrane (Amersham International) with a Bio-Rad Transblot cell at 1 amp overnight. The membrane was blocked in PBS containing 5% skimmed milk powder, 1% FCS, and 0.02% Tween 20 for two hours, and it was then incubated for two hours with 16 μg/mL guinea pig anti-rat M-CSF antibody. After washing, the membrane was incubated with a 1:20,000 dilution of peroxidase-conjugated rabbit anti-guinea pig IgG in PBS containing 1% normal rabbit serum and 0.02% Tween 20. The blot was then developed using the ECL detection kit to produce a chemiluminescent signal, which was captured on x-ray film according to the manufacturer's instructions (Amersham International). Densitometric analysis used the Gel-Pro Analyzer program (Media Cybernetics). No specific bands were seen with omission of the primary antibody. In addition, preincu-

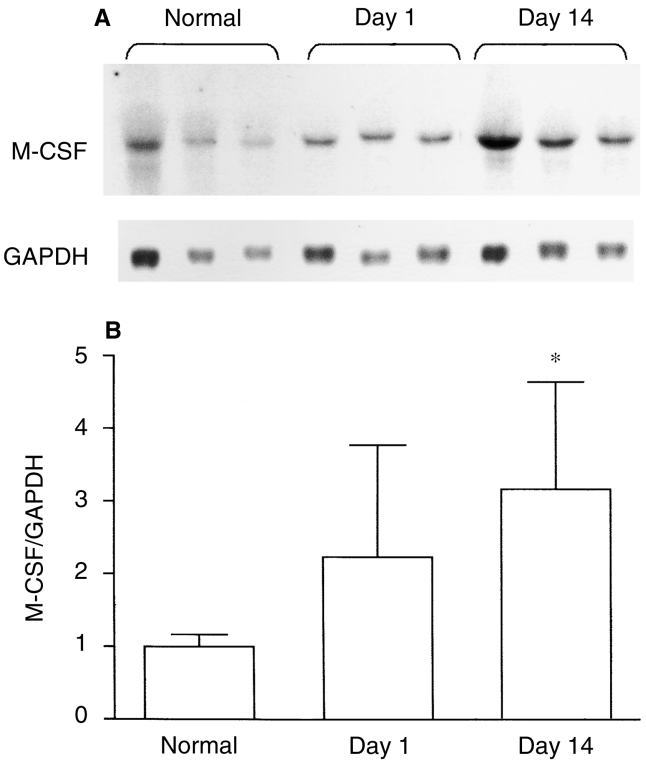

Fig. 1. Northern blot showing up-regulation of macrophage colony-stimulating factor (M-CSF) mRNA in rat anti-glomerular basement membrane (anti-GBM) glomerulonephritis. (*A*) Whole kidney RNA samples from normal rats and on days 1 and 14 of anti-GBM glomerulonephritis were probed for M-CSF and then GAPDH. (*B*) The ratio of M-CSF to GAPDH mRNA. Data are mean ± SD for groups of five animals. $*P < 0.05$ vs. normal by ANOVA using the Bonferroni post-test comparison.

bation of the antibody with 20-fold excess recombinant human M-CSF prevented the detection of specific bands.

Statistics

Statistical analysis, including one-way analysis of variance (ANOVA), was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

M-CSF expression in normal rat kidney

Northern blot analysis identified constitutive expression of a 4 kb band for M-CSF mRNA in normal rat kidney (Fig. 1). In situ hybridization localized M-CSF mRNA expression to small numbers of glomerular cells and approximately 25% of the cortical tubules—mostly distal tubules (Fig. 2a). Medullary tubules were also positive for M-CSF mRNA (data not shown). Immunohistochemistry staining using a purified guinea pig antibody identified constitutive M-CSF expression in a small number of glomerular cells in normal rat kidney (Fig. 3a). In addition, antibody staining showed M-CSF protein

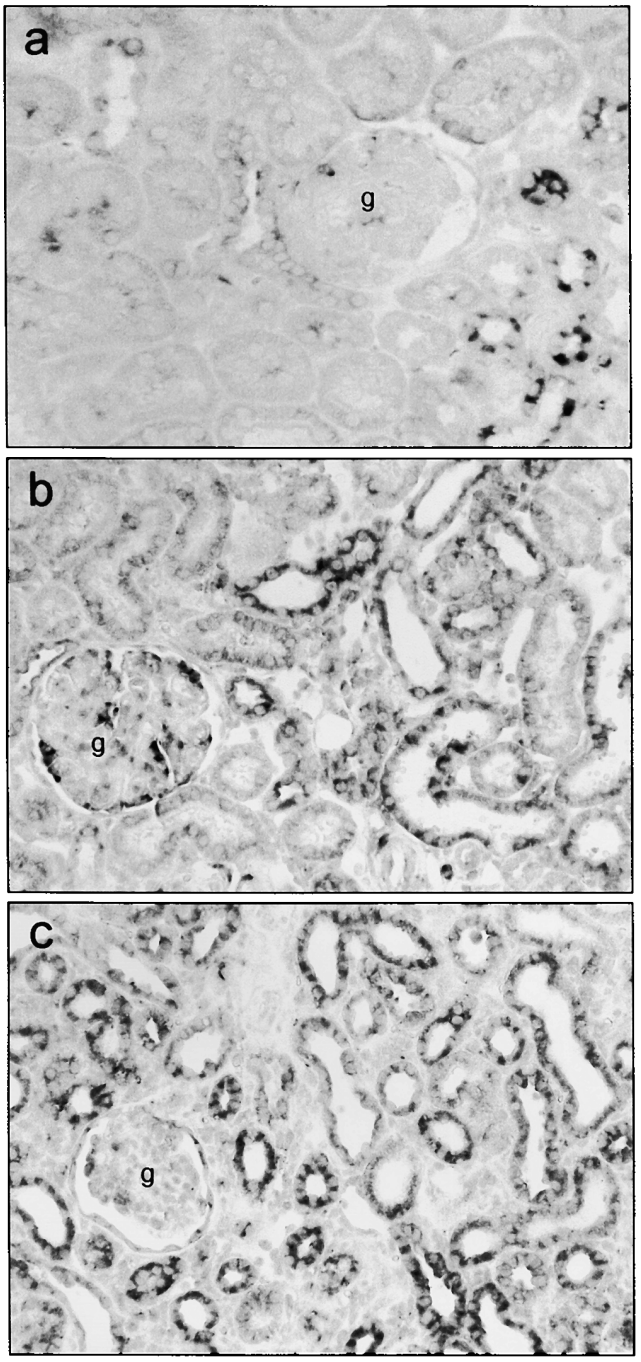

Fig. 2. Localization of M-CSF mRNA expression in normal and diseased rat kidney by in situ hybridization. (*a*) Normal rat kidney showing M-CSF mRNA expression (purple) by some glomerular cells and by a minority of cortical tubules (mostly distal tubules). (*b*) Day 14 of anti-GBM glomerulonephritis showing a marked increase in M-CSF mRNA expression (purple). Many stained cells are apparent in the glomerulus, including cells with a distinctive podocyte morphology. Most cortical tubules are positive for M-CSF mRNA. Some interstitial cells also express M-CSF mRNA. (*c*) An obstructed rat kidney showing increased tubular M-CSF mRNA expression (purple), including the dilated tubules. However, the glomerulus shows M-CSF expression by only a few cells, mostly podocytes. Abbreviation is g, glomerulus. Sections had no counterstain (a) or a methyl green nuclear counterstain (b and c). Magnification ×250.

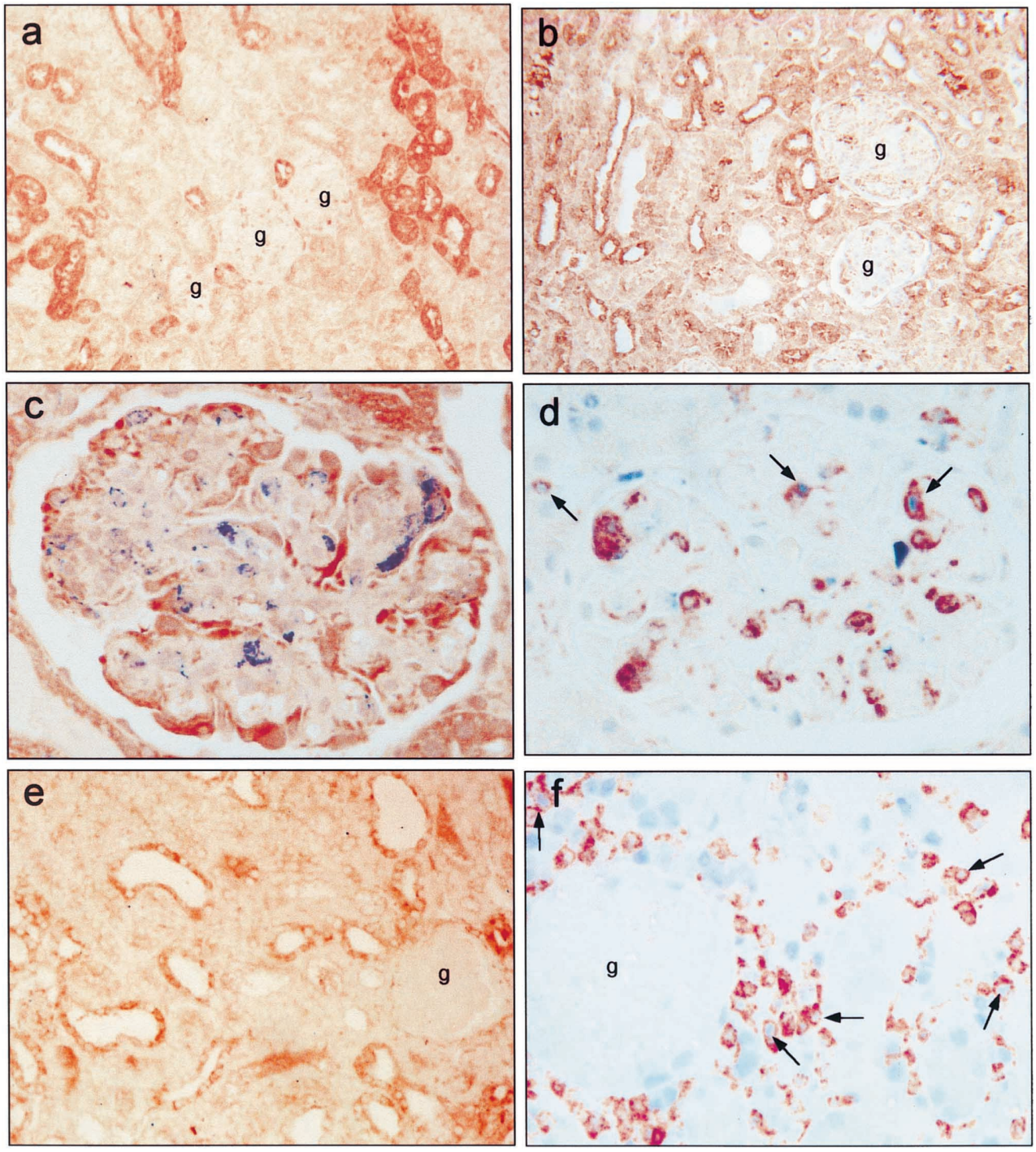

Fig. 3. Localization of M-CSF protein by immunohistochemistry staining in normal and disease rat kidney. (*a*) Normal rat kidney showing M-CSF expression (brown) by a few glomerular cells and by some cortical tubules (mostly distal tubules). (*b–d*) Day 14 of rat anti-GBM glomerulonephritis. (b) An increase in glomerular M-CSF staining is seen. Most striking is the increase in tubular M-CSF staining, with most cortical tubules positive. (c) Glomerulus showing M-CSF staining (brown) by podocytes associated with macrophage infiltration identified by cytoplasmic ED1 staining (blue). (d) Glomerular macrophage accumulation (brown ED1+ cells), some of which are proliferating (blue nuclear PCNA staining, arrows). (*e* and *f*) Obstructed rat kidney. (e) Increased tubular M-CSF staining (brown) is evident in cortical tubules, including dilated tubules. However, no change is evident in glomeruli. (f) Many interstitial macrophages (brown ED1+ cells), some of which are proliferating (blue nuclear PCNA staining, arrows), are seen in the obstructed kidney. Note the lack of glomerular macrophage accumulation. Abbreviation is g, glomerulus. No counterstain was used in (a, d, e, and g). Hematoxylin nuclear counterstain (blue) was used in (b and c). Magnification ×160 (a, b, and e), ×250 (f), ×400 (c and d).

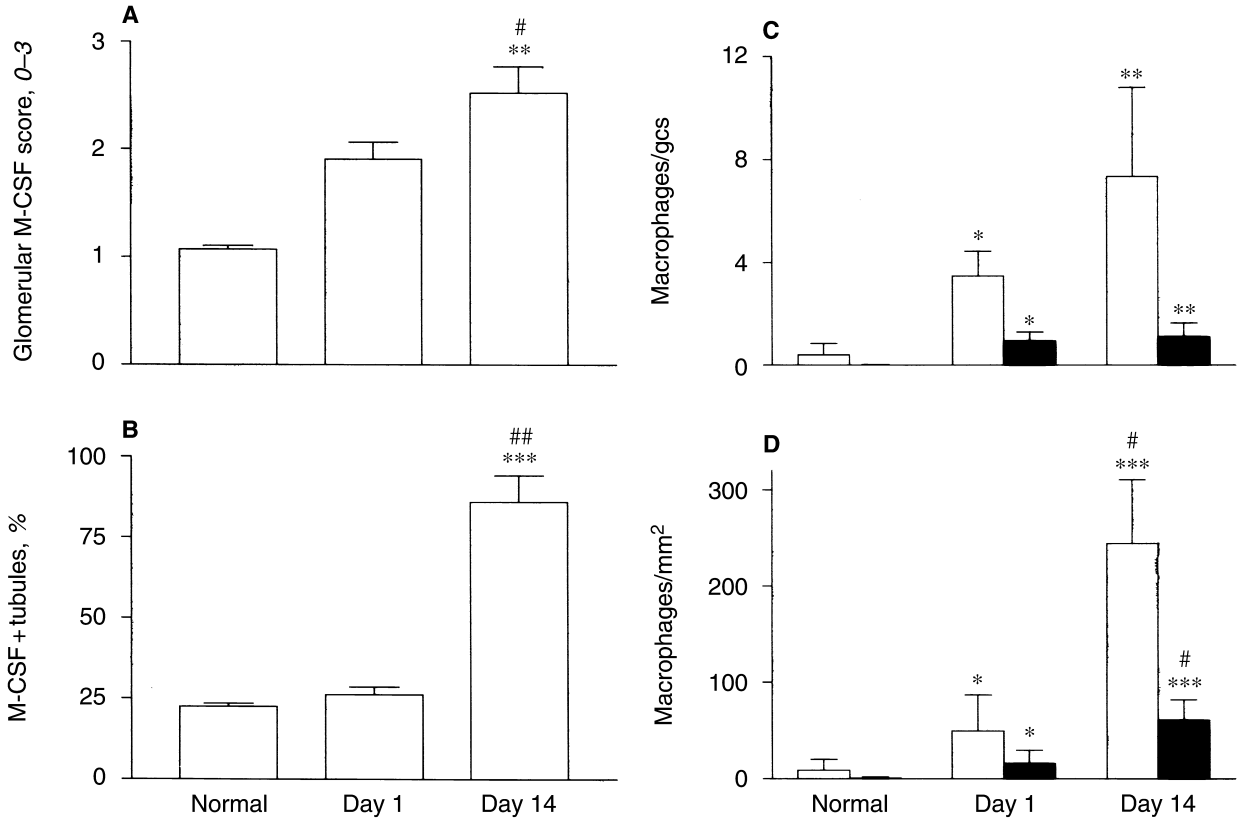

Fig. 4. Quantitation of immunohistochemistry staining for M-CSF expression and macrophage proliferation in rat anti-GBM glomerulonephritis. M-CSF staining in glomeruli (*A*) and cortical tubules (*B*). Staining for total macrophages (ED1+ cells, □) and proliferating macrophages (ED1+ PCNA+ cells, ■) in glomeruli (*C*) and the cortical interstitium (*D*). Data are mean ± SD for groups of five animals. Abbreviation is cgs, glomerular cross-section. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. normal; $\#P < 0.05$; $\#\#P < 0.01$ vs. day 1, by ANOVA using the Bonferroni post-test comparison.

expression by 23% of cortical tubules in a pattern very similar to that seen with in situ hybridization (Figs. 3a and 4). Medullary tubules were also positive for M-CSF immunostaining (data not shown).

The guinea pig anti-rat M-CSF antibody recognized recombinant mouse and human M-CSF as shown by Western blotting (Fig. 5A). Using this antibody, a form of M-CSF of approximately 40 kD was detected in tubules isolated from normal rat kidney (Fig. 5B). Normal rat serum was shown to contain two forms of M-CSF of approximately 40 and 85 kD, consistent with previous studies [30]. M-CSF was not detected in the urine of normal rats (Fig. 5C).

Up-regulation of M-CSF in rat anti-GBM glomerulonephritis

Accelerated anti-GBM glomerulonephritis was induced in SD rats. Animals developed moderate proteinuria (47 ± 7 on day 1, 170 ± 12 on day 7, 130 ± 29 on day 14 vs. 6 ± 1 mg/24 h in normal rats) and a reduction in the rate of creatinine clearance (0.61 ± 0.05 on day 1, 0.22 ± 0.02 on day 7, 0.21 ± 0.05 on day 14, vs. 0.95 ± 0.14 in normal rats). Histologic renal damage was evident on day 14 in terms of glomerular crescent formation (7.0 ± 1.9%) and tubulointerstitial lesions (1.4 ± 0.9).

Northern blotting demonstrated a progressive increase in whole kidney M-CSF mRNA levels in anti-GBM glomerulonephritis, which became significant on day 14 (Fig. 1). In situ hybridization identified an increase in the number of glomerular cells positive for M-CSF mRNA, with prominent M-CSF expression by podocytes (Fig. 2b). Some infiltrating mononuclear cells and occasional mesangial cells also appeared to express M-CSF. The most dramatic change was the increase in tubular M-CSF mRNA expression, with most cortical tubules positive for M-CSF mRNA on day 14 of anti-GBM glomerulonephritis (Fig. 2b). Some interstitial cells, presumably infiltrating leukocytes, were also positive for M-CSF mRNA.

Immunohistochemistry staining demonstrated a similar increase in glomerular and tubular M-CSF protein expression as that seen with in situ hybridization (Figs. 3 and 4). In the glomerulus, M-CSF staining was prominent in podocytes (Fig. 3c). Some glomerular macro-

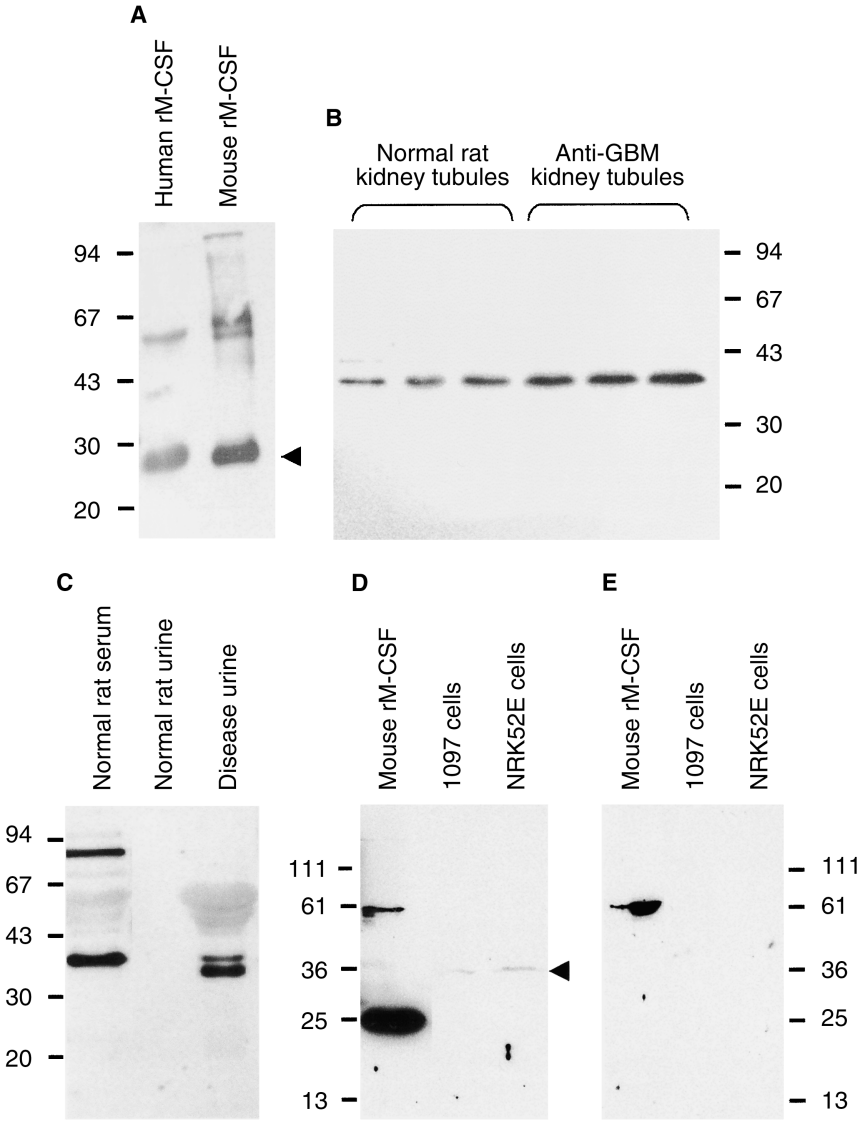

Fig. 5. Western blot detection of M-CSF. (*A*) Detection of mouse and human recombinant M-CSF (rM-CSF). The arrowhead indicates the 26 kD monomer of rM-CSF. (*B*) Equal amounts of tubular lysates (30 μg) were examined from normal rat kidney and on day 14 of anti-GBM glomerulonephritis. A single band of approximately 40 kD was detected, which was increased 2.2-fold in the disease samples. (*C*) Two bands of approximately 40 and 85 kD are seen in normal rat serum. No M-CSF is detected in 10 μL of normal rat urine, but two bands of approximately 37 and 40 kD are seen in 10 μL of urine from day 14 of anti-GBM glomerulonephritis. (*D*) A band of approximately 36 kD (arrowhead) is seen in lysates of rat 1097 mesangial cells and NRK52E tubular epithelial cell lines. (*E*) Absorption of the anti–M-CSF antibody with excess human rM-CSF prevented detection of the 26 kD mouse rM-CSF and of M-CSF in 1097 and NRK52E cells. The remaining upper band in the mouse rM-CSF lane is presumably nonspecific detection of a contaminant. Protein molecular weight markers are indicated.

phages were double stained for M-CSF, but this was difficult to quantitate since the two stains overlapped in the cytoplasm. Staining of mesangial cells was not prominent, being seen in only some glomeruli (data not shown). There was a substantial increase in tubular M-CSF immunostaining, with 86 ± 9% of cortical tubules stained (Figs. 3b and 4). Western blotting demonstrated a 2.2-fold increase ($P < 0.05$ vs. normal) in tubular M-CSF protein on day 14 of anti-GBM disease versus normal kidney (Fig. 5B). Of note, Western blotting demonstrated urinary excretion of M-CSF on day 14 of anti-GBM glomerulonephritis (Fig. 5C).

The increase in glomerular M-CSF expression was closely associated with the accumulation of ED1+ macrophages (Figs. 3c and 4). Similarly, the marked increase in tubular M-CSF expression seen on day 14 of anti-GBM glomerulonephritis was paralleled with interstitial macrophage accumulation at this time (Fig. 4). Double immunostaining demonstrated the presence of ED1+PCNA+ proliferating macrophages in both glomeruli and the interstitium (Figs. 3d and 4). There was a highly significant correlation between the increase in M-CSF expression and the degree of local macrophage proliferation in both the glomerulus and interstitium (Table 1). Glomerular and interstitial M-CSF expression also correlated with the degree of renal dysfunction and urinary protein excretion (Table 1).

Up-regulation of M-CSF in unilateral ureteric obstruction

Five days after ureteric ligation, the left kidney was grossly enlarged and fluid filled. Marked tubular dilation and tubular atrophy were evident together with an inter-

Table 1. Correlation analysis of renal macrophage colony-stimulating factor (M-CSF) expression with macrophage accumulation and proliferation, renal function and proteinuria in rat anti-glomerular basement membrane (anti-GBM) glomerulonephritis

	Glom ED1+	Glom ED1+PCNA+	Interstitial ED1+	Interstitial ED1+PCNA+	Creatinine clearance	Proteinuria
	cells/gcs		*cells/mm²*		*mL/min*	*mg/24 h*
Glomerular M-CSF (0-3)	0.85 ($P = 0.001$)	0.73 ($P = 0.0029$)	—	—	−0.86 ($P < 0.0001$)	0.82 ($P = 0.003$)
M-CSF+ tubules %	—	—	0.93 ($P < 0.0001$)	0.89 ($P < 0.0001$)	−0.78 ($P = 0.0009$)	0.75 ($P = 0.002$)

Data from normal rats and day 1 and day 14 of rat anti-GBM glomerulonephritis were evaluated as one group. Analysis based on glomerular M-CSF score used the Spearman's rank correlation coefficient. Analysis based on tubular M-CSF expression used the Pearson single correlation coefficient. Values shown are the correlation coefficients. Abbreviation is: Glom, glomerulus.

stitial mononuclear cell infiltrate and an increase in interstitial volume.

Northern blotting showed a significant increase in M-CSF mRNA in the obstructed kidney, which was absent in the contralateral control right kidney (Fig. 6). In situ hybridization showed a marked increase in tubular M-CSF mRNA expression in the obstructed kidney, with staining of most cortical tubules, including dilated tubules. However, there was no change in glomerular M-CSF mRNA expression with only a small number of podocytes stained (Fig. 2c). The contralateral control kidney showed no change in M-CSF mRNA expression by in situ hybridization (data not shown). Consistent with mRNA analysis, immunostaining of the obstructed kidney showed an increase in tubular M-CSF protein expression, but no change in glomerular M-CSF expression (Figs. 3e and 7A). No change in tubular M-CSF immunostaining was seen in the contralateral control (Fig. 7A). Accumulation of ED1+ macrophages was evident in the interstitium, but not glomeruli, in the obstructed kidney (Figs. 3f and 7B). No macrophage infiltrate was seen in the contralateral control (Fig. 7B). Double immunostaining identified the presence of ED1+PCNA+ proliferating macrophages within the interstitium of the obstructed kidney, which was absent in the contralateral control (Figs. 3f and 7B).

M-CSF expression by cultured tubular epithelial cells

Tubules were identified as the major site of M-CSF mRNA and protein production in normal and diseased kidney. To explore this issue further, we examine M-CSF production in the rat NRK52E tubular epithelial cell line. NRK52E cells constitutively expressed a 4 kb species of M-CSF mRNA, which was up-regulated 3.1-fold by stimulation with IL-1 (Fig. 8). Western blotting of cell lysates confirmed that NRK52E express M-CSF protein, which was approximately 36 kD, being a similar size to M-CSF detected in lysates of freshly isolated tubules and a cultured rat mesangial cell line (Fig. 5 B, D). As a specificity control, absorption of the anti–M-CSF antibody with excess human recombinant M-CSF was shown to inhibit detection of M-CSF in 1097 and NRK52E cells (Fig. 5E).

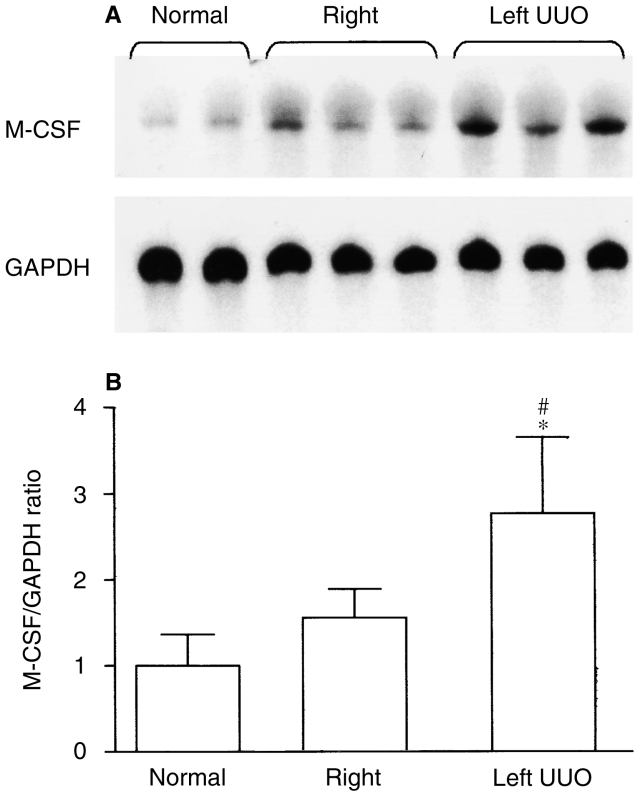

Fig. 6. Northern blot showing up-regulation of M-CSF mRNA in the obstructed rat kidney. (*A*) Whole kidney RNA samples from normal rats and from the contralateral control (right) and the obstructed (left UUO) kidney. (*B*) The ratio of M-CSF to GAPDH mRNA. Data are mean ± SD for groups of five animals. *$P < 0.05$ vs. normal; #$P < 0.05$ vs. right control, by ANOVA using the Bonferroni post-test comparison.

DISCUSSION

This study demonstrates that tubules are the major site of M-CSF production in experimental kidney disease. The implications for M-CSF regulation of local macrophage proliferation and a comparison with previous studies of renal M-CSF expression are considered later in this section.

Examination of two distinct models of kidney disease found a close association between up-regulation of M-CSF expression and macrophage accumulation and local proliferation. In rat anti-GBM glomerulonephritis,

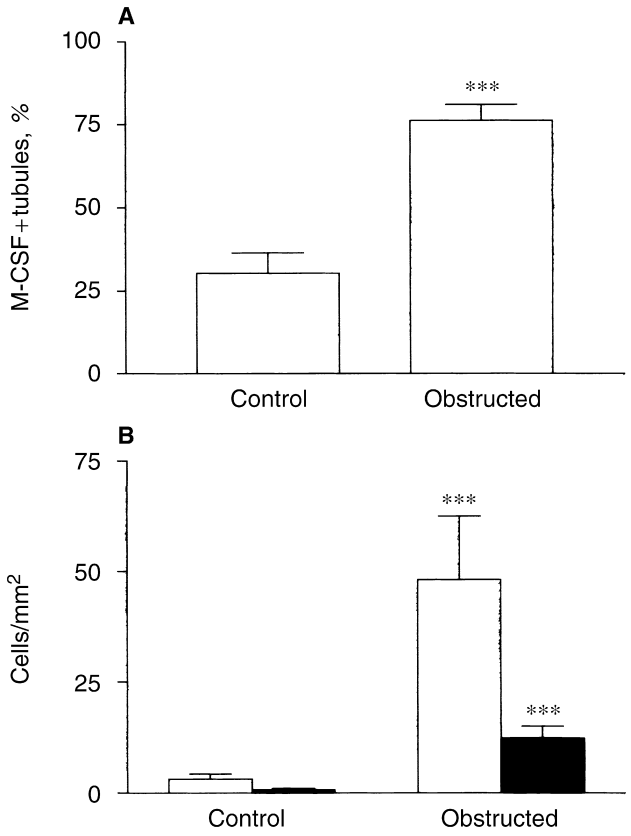

Fig. 7. Quantitation of immunohistochemistry staining for M-CSF expression and macrophage proliferation in the obstructed (left) versus the contralateral control (right) rat kidney. (*A*) M-CSF staining in cortical tubules. (*B*) Staining for total macrophages (ED1+ cells, □) and proliferating macrophages (ED1+PCNA+ cells, ■) in the cortical interstitium. Data are mean ± SD for groups of five animals. Abbreviation is gcs, glomerular cross-section. ***$P < 0.001$ vs. contralateral control by *t* test.

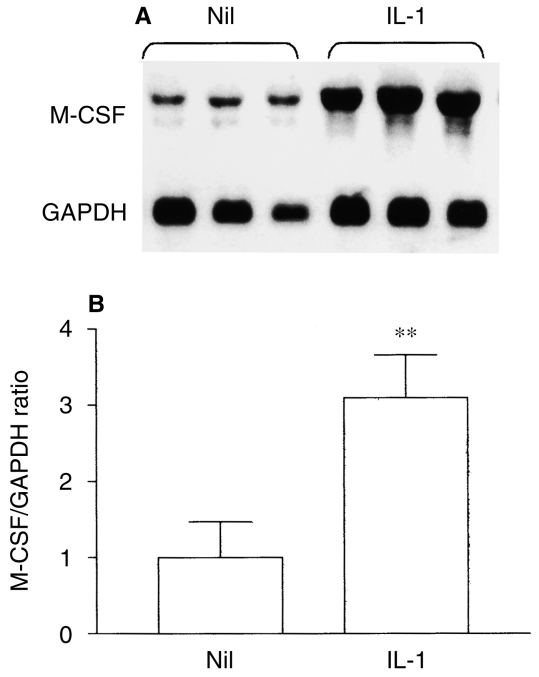

Fig. 8. Northern blot of M-CSF mRNA expression by cultured NRK52E rat tubular epithelial cells. Cells were cultured in medium alone (Nil) or with 10 U/mL IL-1 for six hours and were then RNA analyzed. (*A*) Northern blot showing M-CSF and GAPDH mRNA expression. (*B*) Ratio of M-CSF to GAPDH mRNA. **$P < 0.001$ vs. Nil by *t* test.

there was a progressive increase in glomerular and tubular M-CSF expression that paralleled the development of macrophage accumulation and proliferation in both compartments. The increase in renal M-CSF expression correlated not only with local macrophage proliferation, but also with the degree of renal dysfunction and proteinuria. The obstructed rat kidney also showed a marked increase in tubular M-CSF expression in association with interstitial macrophage accumulation and proliferation. This study has demonstrated, to our knowledge for the first time, that local macrophage proliferation can occur in the absence of glomerular inflammation. Taken together, these results provide strong support for the postulate that increased renal M-CSF production plays an important role in promoting local macrophage proliferation and thereby amplifying macrophage-mediated renal injury. However, final proof for this concept requires functional blocking studies.

Tubular epithelial cells were identified as the major site of M-CSF production in the two models of kidney disease examined. This was demonstrated by three independent methods: in situ hybridization, immunostaining, and Western blotting. Consistent with these findings, Northern blotting showed an up-regulation of M-CSF mRNA in both disease models. In a separate study, we also used immunostaining to show a marked up-regulation of tubular M-CSF expression in various forms of human glomerulonephritis, which correlated with local macrophage proliferation and the degree of renal dysfunction (Isbel et al, *Nephrol Dial Transplant*, manuscript in press).

The finding that IL-1 caused an increase in M-CSF mRNA levels in cultured NRK52E tubular epithelial cells provides an attractive explanation for the increased in tubular M-CSF mRNA and protein expression seen in rat anti-GBM glomerulonephritis. We have previously demonstrated that IL-1β mRNA and protein expression is up-regulated in both the glomerulus and tubules in this disease model [31]. Blockade of IL-1 activity in this disease model causes a reduction in glomerular, and most particularly, tubulointerstitial macrophage accumulation and proliferation [32, 33]. Thus, up-regulation of renal M-CSF production may be an important pathogenic mechanism of IL-1 induced renal injury.

In a study of MRL-*lpr* lupus-prone mice, Wada et al identified an increase in glomerular M-CSF expression in

association with renal disease by immunohistochemistry staining using a MoAb, but no tubular staining was evident despite the fact that aggressive interstitial macrophage accumulation develops in this disease model [18]. The apparent discrepancy with the current study may be due to differences in the epitopes of the M-CSF molecule recognized by the respective antibodies. The single epitope recognized by the monoclonal anti-M-CSF antibody in glomerular cells may not be present in the tubular form of M-CSF due to differences in glycosylation or alternative mRNA splicing, which are mechanisms known to contribute to the heterogeneity of the M-CSF molecule [11, 34]. The current study used a polyconal antibody that presumably recognizes multiple epitopes within the M-CSF molecule and thus is more likely to recognize multiple forms of M-CSF. This possibility is supported by an in situ hybridization study of MRL-*lpr* mice in which weak tubular M-CSF mRNA staining was reported during the development of lupus nephritis [35]. A similar explanation may account for the differences with the study by Matsuda et al in which immunostaining identified glomerular, but not tubular, M-CSF staining in a study of IgA nephropathy [19].

An increase in urinary M-CSF excretion was consistently observed in rat anti-GBM glomerulonephritis. Urinary M-CSF was similar in size to that produced by tubules and mesangial cells, suggesting that secretion by renal cell types may contribute to urinary M-CSF excretion. Alternatively, urinary M-CSF could be derived from circulating M-CSF. Increased urinary M-CSF excretion has also been described in IgA nephropathy [36], although no assessment of macrophage accumulation or proliferation within the kidney was made.

In summary, this study clearly demonstrates that tubular epithelial cells are the major sites of M-CSF production in experimental kidney disease. In particular, substantial macrophage accumulation and local proliferation occur in the tubulointerstitium in the absence of glomerular inflammation. These results suggest that M-CSF production within the kidney, and particularly tubular M-CSF production, plays an important role in promoting local macrophage proliferation in experimental kidney disease.

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